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(54) Title: HEPARIN-PRODUCING MURINE MASTOCYTOMA CELL LINES

(57) Abstract

Cell lines which can be grown continuously in culture and which provide glycosaminoglycan (GAG) compositions with high antiproliferative activity with respect to smooth muscle cells are disclosed. These cell lines permit the production of large amounts of antiproliferative GAGs which also lack antithrombin-binding activity. In addition, methods to obtain mutant cell lines which produce GAG compositions with desired activity spectra are disclosed.

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HEPARIN-PRODUCING MURINE MASTOCYTOMA CELL LINES

Technical Field

The invention relates to the field of complex polysaccharides and methods to produce them. In particular, it concerns stable cell lines useful for producing antiproliferative polysaccharides and methods to obtain them.

Background Art

Glycosaminoglycans (GAG) are copolymers of alternating hexosamine and aldouronic acid residues which are found in sulfated forms and are synthesized as proteoglycans. They have collectively been called mucopolysaccharides, and various common names apply to members of this class, according to the nature of the hexosamine/aldouronic acid repeating units. In chondroitin sulfates, the aldouronic acid is D-glucuronic acid, and the hexosamine is acetylated 2-amino-2-deoxy-D-galactose (N-acetyl galactosamine, GalNAc). In dermatan sulfate (chondroitin sulfate B) some of the aldouronic acid is L-iduronic acid and the hexosamine is GalNAc. In keratan sulfate, the aldouronic acid is replaced by D-galactose, and the hexosamine is mostly acetylated 2-amino-2-deoxy-D-glucose

(N-acetyl glucosamine, GlcNAc). In heparan sulfate and heparin, the hexosamine is mostly acetylated and sulfated glucosamine ($GlcNH_2$), and the aldouronic acid is mostly L-iduronic in heparin and mostly D-glucuronic acid in heparan sulfate. Thus, heparan sulfate, in general has a higher proportion of glucuronic acid than heparin.

Problems of heterogeneity in preparations of heparan sulfate or heparin isolated from tissues make sharp distinctions difficult, since these oligosaccharides are related by their biosynthetic pathway. Conventional heparin (used as an anticoagulant) has a molecular weight of 5-25 kd and is extracted as a mixture of various chain lengths by conventional procedures. These procedures involve autolysis and extraction of suitable tissues, such as beef or porcine lung, intestine, or liver, and removal of nonpolysaccharide components.

The molecular weight of the chains in the extract is significantly lower than the 60-100 kd known to exist in the polysaccharide chains of the heparin proteoglycan synthesized in the tissue. The GAG moiety is synthesized bound to a protein core at a serine residue through a tetrasaccharide linkage region of the sequence -D-GlcA-Gal-Gal-Xyl-(SER), which is then elongated at the D-glucuronic residue with alternate additions of GlcNAc and D-glucuronic acid. The polysaccharide side-chains are modified by a series of enzymes which sequentially deacetylate the N-acetyl glucosamine and replace the acetyl group with sulfate, epimerize the hydroxyl at C5 of the D-glucuronic acid residue (to convert it to L-iduronic acid), sulfate the O-2 of the resulting L-iduronic acid, and then sulfate the O-6 of the glucosamine residue. Some of the chains are further sulfated at the O-3 of the glucosamine residue. 3-O-sulfation can occur in heparan or heparin. This further sulfation is associated with the active site for antithrombin (anticlotting) activity. Other chemically possible sulfation sites are on the O-3

of L-iduronic or D-glucuronic and O-2 of D-glucuronic acid. In general, heparin has a greater content of sulfated glucosamine than heparan and also a higher content of L-iduronic acid.

Due to their obvious chemical similarity, isolated "heparin" chains may contain regions of what might otherwise be classified as heparan sulfate.

The inherent variability and heterogeneity of heparin or heparan sulfate preparations from animal tissues, as well as the irreproducible nature of these preparations, makes the use of standard animal sources for particular polysaccharide components disadvantageous. It would be far preferable to obtain a clonal source which would permit a dependable and relatively invariant reservoir for these moieties. In particular, it is known that heparin and heparan sulfate have a variety of biological activities including activities as anticoagulants, inhibitors of smooth muscle cell proliferation and of antiinflammatory activity in a variety of biological functions, depending on the precise composition of the material (see, for example, European Patent Application 287477). Utilization of a clonal source for these materials would permit production of heparin/heparan sulfate with a reproducible spectrum of these activities, as well as providing adequate supplies.

Of particular interest with regard to the cell lines described below are heparin preparations having antiproliferative effects on smooth muscle.

Proliferation of smooth muscle cells in blood vessel walls occurs in response to vascular injury and in association with certain disease states (Austin, G.E., et al., J Am Coll Cardiol (1985) 6:369-375). The proliferation of these cells can have negative effects due to the production of excess proteins or other matrix molecules, which, along with the cells themselves, form pathologic lesions of, for example, atherosclerosis, renal hyper-

tension, pulmonary hypertension, vasculitis, and post-surgical vascular retinosis. These results are distinguished from the acute response to trauma characterized by blood clotting.

The involvement of heparin or heparan sulfate in smooth muscle proliferation has been recognized for some time. Heparin and heparan sulfate can slow or arrest the vascular proliferation associated with injury described hereinabove (Clowes, A.W., et al., Nature (1977) 265:625-626). The effect of heparan sulfate and heparin on smooth muscle proliferation is also described by Marcum, J.A., et al., in Biology of Proteoglycan, Academic Press (1987), pp. 301-343. The inhibition of vascular smooth muscle cell growth by heparin was further described by Castellot, J.J. Jr., et al., J Biol Chem (1982) 257:11256-11260, and the effect of heparin on vascular smooth muscle cell growth in fetal tissue was described by Benitz, W.E., et al., J Cell Physiol (1986) 127:1-7. The effect of heparin as an inhibitor of both pericyte and smooth muscle cell proliferation was shown by Orlidge, A., et al., Microvascular Research (1986) 31:41-53, and these authors further showed that chondroitin sulfate and dermatan sulfate do not have this effect. Wright, T.C., et al., J Biol Chem (1989) 264:1534-1542, showed that heparin and oligosaccharides derived from heparin inhibited growth of smooth muscle cells and rat cervical epithelial cells. A review of the effects of heparin and heparan sulfate on the proliferation of smooth muscle cells is in press (Benitz, W.E. in "The Pulmonary Circulation: Normal and Abnormal", Fishman, A.P., ed., University of Pennsylvania Press (1988)).

It is not clear by what mechanism these glycosaminoglycans operate, or to what extent they interact with other growth factors such as epithelial and fibroblast growth factors. It has been proposed that a 3-O sulfate on an oligosaccharide of at least 5 sugars may be

important in this process (Castellot et al., J Cell Biol (1986) 102:1979-1984).

The present invention provides a reliable and stable source of heparin with antiproliferative activity and a method for obtaining alternate clonal sources of glycosaminoglycans with reproducible spectra of biological activities.

Disclosure of the Invention

The invention is directed to cell lines derived from murine mastocytoma cells. Some cell lines have been selected for their ability to adhere to plastic surfaces. These cell lines secrete a heparin composition which is antiproliferative and relatively lacking in antithrombin-binding activity. The heparin composition is synthesized by these cells despite numerous passages. These and other GAG-producing cells can be screened in tissue culture using a polyester overlay technique to detect clones that produce altered forms of GAG. The cell lines of the invention offer a method to produce, in quantity, polysaccharide compositions which have a variety of biological activities, including inhibition of smooth muscle cell proliferation.

In one aspect, the invention is directed to immortal cell lines derived from a suspension of murine mastocytoma cells. These cell lines synthesize a polysaccharide mixture consisting of about 15% chondroitin sulfate and about 85% heparin/heparan sulfate, and the heparin/heparan sulfate fraction of this composition is antiproliferative while having minimal antithrombin-binding activity. The invention is also directed to the heparin/heparan sulfate fraction of this polysaccharide mixture.

In another aspect, the invention is directed to immortalized cell lines making the glycosaminoglycan of the above-mentioned composition, which cell lines have

been selected for adherence to plastic surfaces. In another aspect, the invention is directed to a method to produce and screen mutant colonies which are defective or effective in GAG synthesis, which comprises inducing colony formation from single adherent cells on tissue culture plates overlaid with one or more disks of polyester cloth so as to result in a transfer of cells from the colonies to the cloth, and then assessing the amount and/or type of GAG synthesized by the transferred cells on the cloth.

In another aspect, the invention is directed to pharmaceutical compositions containing the heparin/heparan sulfate compositions exhibiting antiproliferative or other biological activity and to methods to prevent the proliferation of cells or produce other biological effects using these compositions.

In still another aspect, the invention is directed to antibodies reactive with the antiproliferative heparin/heparan sulfate.

Brief Description of the Drawings

Figure 1 shows the elution pattern for the invention heparin on anion-exchange HPLC.

Modes of Carrying Out the Invention

By "heparin/heparan sulfate" is meant a preparation obtained from the cell line and having the characteristics of this mixture, separated from the chondroitin sulfate also produced. This preparation may include residues of D-glucuronic acid (GlcA) as characteristic of heparan sulfate, as well as iduronic acid (IdoA) as characteristic of heparin. As described in the Background section above, the conversion of D-glucuronic acid to L-iduronic acid is a result of epimerization at the 5 carbon in a heparan-type intermediate. To the extent that full conversion has not been

made, heparan sulfate characteristics remain in the preparation. Because the precise nature of the polymeric chains in the preparations of heparin is not generally determined and varies from preparation to preparation, the term "heparin/heparan sulfate" is intended to cover the range of mixtures encountered. In the case of GAG compositions derived from a single clonal source, the composition of the GAG is reproducible but also contains components which can be considered either heparin or heparan sulfate. Accordingly, this designation is appropriate for such reproducible compositions as well.

Over 90% of the heparin/heparan sulfate made by the cell lines is stored inside the cells within granules. A crude preparation of cellular heparin/heparan sulfate can be generated by triggering the cells to secrete the mixture with a calcium ionophore or with an IgE antibody and appropriate antigen. Alternatively, the cells can be separated from the growth medium by centrifugation or filtration and treated with alkali, proteases, nucleases, and detergent to remove nonpolysaccharide contaminants. The heparin/heparan sulfate is then collected by anion-exchange chromatography and solvent precipitation. Chondroitin sulfate is removed by digestion with chondroitinase.

Continuous Cell Lines Which Produce Antiproliferative Compositions

The cell lines which comprise one aspect of the invention are prepared from suspensions of cells derived from a murine mastocytoma tumor originally described by Furth, J., et al., Proc Soc Exp Biol (NY) (1957) 95:824-828. Although this tumor has been serially passaged through mice since its original description and has continuously produced heparin in this in vivo context, previously obtained suspensions of cells derived from the

tumor have lost the ability to produce heparin over a period of several months.

To produce the cell lines of the invention which maintain the ability to produce heparin in culture, a suspension of these tumor cells is treated to remove macrophages and fibroblasts according to the procedure of Jacobsson, K.G., et al., J Biol Chem (1985) 260:12154-12159. The suspension is then treated with streptomycin, penicillin, gentamycin and Fungizone to remove contaminating bacteria and fungi, and the cells are then cultured for three days with tetracycline. Standard growth conditions for the cells are RPMI 1640 growth medium supplemented with 15% heat-inactivated fetal bovine serum and 100 ug/ml of penicillin G at 37°C under an atmosphere of 5% CO_2 in 95% air. The cells also divide in other types of growth media, performing especially well in formulations of modified Eagle's medium supplemented with as little as 0.5% heat-inactivated fetal bovine serum and 1% bovine serum albumin. Under these conditions the cells divide every 12-15 hours and achieve a density of 1-2 x 10^6 cell per ml. When this density of cells is achieved, they stop growing, but when diluted with fresh growth medium, they resume exponential growth. The cells can be frozen under liquid nitrogen in growth medium containing 8% glycerol as cryoprotectant and can be recultured in portions when thawed. Preparations of cells derived from a parental line prepared in this manner, and designated MST herein, can be recultured, and they maintain their ability to produce heparin for over six months.

In addition, cell suspensions derived from a variety of animal mastocytomas, such as human, murine, canine and equine mastocytomas, are treated in the manner described above and can be selected for adherence. The production of adherent colonies permits facile assessment of the effects of mutagenesis on the production of glycosaminoglycans and does not itself diminish the

capability of the cells to produce GAG compositions with antiproliferative activity. Selection is made by culturing the mastocytoma-derived cell suspensions in plastic cell culture flasks in the culture medium described above, removing unattached cells by replacing the medium every 4 to 7 days, and washing the subset of attached cells to remove loosely adherent cells. By this selection process, a confluent monolayer of firmly attached cells is generated after 4-6 weeks. The adherent cells are treated with, for example, 0.125% trypsin and 1 mM EDTA to release them as a single cell suspension which can be frozen in liquid nitrogen as described above.

Populations of adherent cells prepared from the MST parent suspension described above are designated MTA. These lines also maintain the ability to produce antiproliferative heparin compositions for over three months when passaged in culture.

The cell preparations described above, such as the MST suspension or an adherent line, can be subcloned into unique clonal cell lines by limiting dilution in microtiter dishes. These cloned cell lines continue to produce heparin with antiproliferative activity.

Heparin can be isolated from the cultures of either the mixed suspensions or the cloned cell lines as described above (see Example 2). The heparin preparations from these cells, when analyzed by anion-exchange HPLC on TSK-DEAE-3SW, eluted at salt concentrations slightly greater than that required to elute commercial heparin, suggesting a high degree of sulfation. Nitrous acid degradation shows that about 80% of the glucosamine residues are N-sulfated.

In addition, the nature of the GAG composition can be determined by culturing the cells in the presence of radiolabeled polysaccharide precursors followed by digestion of the resulting radioactive GAGs using chondroitinase ABC. This analysis demonstrates that the

cultures or cell lines obtained in this manner contain about 10-25% chondroitin sulfate and about 75-90% heparin.

A typical cell line exemplary of the adherent cells prepared by the invention was designated MTA-1 and was deposited at the American Type Culture Collection on _____ and has ATCC No. _____. This cell line grows continuously in culture and produces heparin/heparan sulfate in a stable manner. The heparin showed antiproliferative activity with respect to cultured smooth muscle cells from blood vessels using an assay described below, and was low in antithrombin-binding activity. The cell line produces about 0.5 ug uronic acid equivalents of heparin per 10^6 cells; thus, quantities of heparin polysaccharide greater than 100 mg can conveniently be produced.

An outline of this work was described by Montgomery, R.I., et al., J Cell Biol (1988) 107:157A, incorporated herein by reference.

Mutagenesis and Screening

The production of adherent mastocytoma cells as described above facilitates the mutagenesis and screening of mutants which produce desired GAG compositions. In this procedure, adherent cells are treated with mutagens such as methyl methane sulfonate, ethyl methane sulfonate, N-methyl-N'-nitro-N-nitrosoguanidine, or nonchemical mutagens such as gamma radiation or ultraviolet light to produce alterations. The effectiveness of mutagenesis can be assessed by scoring mutations in standard marker genes, such as the Na^+/K^+ATPase gene or the hypoxanthine phosphoribosyl transferase gene. Mutations in these genes render the cells resistant to the drugs ouabain and 6-thioguanine, respectively. Cultures which have a high level of mutation are then subjected to replica-plate screening to assess their GAG or heparin/heparan sulfate production.

The adherent cell lines can also be transfected with DNA that encodes core proteins or enzymes that enable the cells to produce heparin/heparan sulfate. The cells can also be fused with other cell lines that produce unusual forms of heparin/heparan sulfate that have different biological properties from the forms of heparin/heparan sulfate produced by the mastocytoma cells. These transfected and hybrid cell lines can be replica plated using the polyester overlay technique which enables the identification of specific clones with desired heparin/heparan sulfate compositions and biological properties.

The replica-plating method is described in detail by Esko, J.D., Meth in Cell Biol (1989) 32:387-422, incorporated herein by reference. In this review, the methods of replica plating of animal cells are considered in general, including assessment of the replicas for heparan sulfate and proteoglycan synthesis in general (Esko, J.D., et al., Proc Natl Acad Sci (USA) (1985) 82:3197-3201; Esko, J.D., et al., J Biol Chem (1986) 261:15725-15733; Esko, J.D., et al., J Biol Chem (1987) 262:12189-12195; Bame, K.J., and Esko, J.D., J Biol Chem (1989) 264:8059-8065; and Esko, J.D., et al., Science (1988) 241:1092-1096).

Although a variety of absorbents for replica plating is disclosed in this review, polyester cloth is preferred as an absorbent for cells derived from colonies which are to be assessed for the production of GAGs. Most preferably, a double layer of polyester cloth wherein the lower layer has a larger pore size than the upper layer is used.

The cloth layers can be subjected to in situ assays for production of GAGs. For example, the layers are incubated in growth medium containing radioactive sulfate for several hours, then treated with trichloroacetic acid to precipitate radioactive heparin/heparan sulfate proteoglycans. Unincorporated sulfate is

then removed by washing, for example on a Buchner funnel, with additional acid, and the incorporation of the radioactive sulfate into the precipitated GAGs is assessed by detecting label, for example with X-ray film. Because the cells are adherent, representative colonies remain on the culture plate and can be matched to the results on the absorbing cloth.

Another strategy for isolating GAG mutants deficient in sulfating the polysaccharide is based on radiation suicide. A suspension of mutagen-treated cells is incubated with radioactive sulfate, and the labeled cells are stored under liquid nitrogen. Over 4-6 weeks the cells that incorporated radioactive sulfate into heparin/heparan sulfate die due to the decay of the radioactive sulfate. Thus, the surviving population of cells is enriched for mutants that are unable to make heparin/heparan sulfate with a high level of sulfation. Individual mutants can be identified and cloned by replica plating as described above. Although the GAG produced has a low sulfation level, it may have useful biological properties altered from those of the GAG produced in wild type. This screening method has the advantage that it operates by selection.

The cloth absorbents can be subjected to extensive analysis for the nature of the GAGs produced and for the quantities obtained. Colonies established on polyester cloth can be tested for antithrombin-binding activity, antiproliferative activity, antiinflammatory activity, growth factor binding activity, and other properties of the GAG produced. These properties can be assessed on a colony-by-colony basis and matched to the growing colonies on the culture plates. For example, colonies on the disks can be gently lysed to release the stored heparin/heparan sulfate. The disks can then be incubated with radioactively labeled antithrombin, and colonies that produce a heparin/heparan sulfate composi-

tion that has the antithrombin-binding sequence will bind the radioactive antithrombin. Exposure of the disk to X-ray film permits the detection of clones that produce antithrombin-binding heparin/heparan sulfate. Another technique that can be used to isolate variants that produce more potent antiproliferative heparin/heparan sulfate involves overlaying the colonies on the disks with a thin layer of agar or polyacrylamide. Smooth muscle cells are then placed on top of the agar or acrylamide and permitted to proliferate. Cells situated above mastocytoma clones that produce more potent antiproliferative heparin/heparan sulfate will grow more slowly than cells above colonies that produce less active heparin/heparan sulfate, resulting in a clear zone. Similarly, cells above clones that have lost the ability to produce antiproliferative heparin/heparan sulfate will grow more rapidly, resulting in a zone of heavy cell growth.

In this manner, mutants are obtained which produce GAGs and heparin/heparan sulfate fractions having the desired biological functions in a reproducible manner.

Preparation of Antibodies

The compositions containing predominantly heparin/heparan sulfate separated from the cell cultures herein can be used to stimulate the production of antibodies which immunoreact with the components of the composition. Standard immunization protocols using this composition in various mammals, such as rabbits, rats, mice, and sheep, result in antisera which are immunoreactive with the composition components. The composition may be advantageously conjugated to a suitable antigenically neutral carrier, such as an appropriate serum albumin or keyhole limpet hemocyanin, in order to enhance immunogenicity. Furthermore, the antibody-secreting cells of the immunized mammal can be immortalized to generate monoclonal antibody

panels which can then be screened for reactivity with the composition. In vitro immunization procedures can be used to produce human monoclonal antibodies and antibodies to polysaccharide antigens that are weakly immunogenic (Reading, C.L., Meth in Enzymol (1986) 121:18-27; Boss, B.D., Meth in Enzymol (1986) 121:27-33; Borrebaeck, C.A.K., and Moller, S.A., J Immunol (1986) 136:3710-3715.) Techniques for preparation of mouse monoclonal antibodies to heparin subunits are described by Pejler, G., et al., J Biol Chem (1988) 263:5197-5201, incorporated herein by reference.

The resulting polyclonal or monoclonal antibody preparations are useful in assays for levels of the corresponding heparin/heparan sulfate, e.g., the active antiproliferative component, in biological samples, as described below. The antibodies raised against, e.g., antiproliferative heparin/heparan sulfate compositions can also be used in passive therapy to prevent excessive repression of the proliferation of smooth muscle cells.

Statement of Utility

The compositions of the invention containing predominantly heparin/heparan sulfate are useful in therapeutic applications for treatment of various conditions or diseases. For example, compositions which show antiproliferative activity are useful for conditions which are characterized by excessive and destructive smooth muscle cell proliferation. These conditions frequently occur where the subject has been exposed to trauma, such as in the case of surgical patients. The trauma caused by wounds or surgery results in vascular damage, and secondary smooth muscle cell proliferation results in vascular retinosis. This undesirable result can occur after vascular graft surgery, heart transplantation, balloon or laser angioplasty, arterial traumatic injury, postsurgical repair of muscular arteries, long-term in-dwelling of

arterial catheters, invasive arterial diagnostic procedures, kidney, lung or liver transplants, coronary artery bypass surgery, carotid artery bypass surgery, femoral popliteal bypass surgery, and intracranial arterial bypass surgery.

In addition to secondary smooth muscle cell proliferation events occurring as a result of trauma, certain diseases are associated with unwanted vascular proliferation, although in these cases, too, it is assumed that some internal unknown injury has caused the secondary result. These disease states include Goodpasture syndrome, acute glomerulonephritis, neonatal pulmonary hypertension, asthma, congestive heart failure, adult pulmonary hypertension, and renal vascular hypertension.

For all these diseases and conditions, administration of suitable amounts of the antiproliferative compositions of the invention is useful in treatment. Administration is by typical routes appropriate for polysaccharide compositions, and generally includes systemic administration, such as by injection. Particularly preferred is intravenous injection, as continuous injection over long time periods can be easily continued. Typical dosage ranges are in the range of 0.1-10 mg/kg/hr on a constant basis over a period of 5-15, preferably 7-10, days. Particularly preferred dosage is about 0.5 mg/kg/hr, or, for a 70 kg adult, 35 mg/hr or 840 mg/day.

Other modes of administration are less preferred but may be more convenient. Injection subcutaneously at a lower dose or orally at a slightly higher dose than intravenous injection, or by transmembrane or transdermal or other topical administration for localized injury, may also be effective. Localized administration through a continuous release device, such as a supporting matrix, perhaps included in a vascular graft material, is

particularly useful where the location of the trauma is accessible.

Formulations suitable for the foregoing modes of administration are known in the art, and a suitable compendium of formulations is found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA, latest edition.

The antiproliferative compositions of the invention may also be labeled using typical methods such as radiolabeling, fluorescent labeling, conjugation with chromophores or enzymes and used in a competitive assay for the amount of antiproliferative component in a biological sample. Suitable protocols for competitive assays of analytes in biological samples are well known in the art, and generally involve treatment of the sample, in admixture with the labeled competitor, with a specific finding partner which is reactive with analyte such as, typically, an immunoglobulin or fragment thereof. The antibodies prepared according to the invention are useful for this purpose. The binding of analyte and competitor to the antibody can be measured by removing the bound complex and assaying either the complex or the supernatant for label. The separation can be made more facile by preliminary conjugation of the specific binding partner to a solid support. Such techniques are well known in the art, and the protocols available for such competitive assays are too numerous and too well known to be set forth in detail here.

The antibodies of the invention are useful in immunoassays, not only of the type described above involving competition between labeled composition and the analyte antiproliferation factor in the sample, but also for direct immunoassay for the factor. Alternate protocols involving direct assays are also of wide variety and well known. Typically, the analyte bound to antibody is detected by means of an additional reactive partner which

bears a label or other means of detection. Thus, in typical sandwich assays, for example, the binding of the antibodies of the invention to analyte can be detected by further reaction with a labeled preparation of these same antibodies or by labeled antibody immunoreactive with this preparation by virtue of species differences.

The antibodies of the invention can also be formulated into pharmaceutical compositions and used to stimulate the growth of smooth muscle cells in subjects for which this result is desirable.

Monoclonal antibodies against antiproliferative heparin/heparan sulfate can be used to produce a second generation of antibodies which recognize the binding site for the carbohydrate within the original monoclonal antibody. These second generation antibodies are called internal image antibodies or antiidiotypic antibodies, and they mimic the structure of the original carbohydrate antigen. Such antibodies should have the properties of the original antigen and thus would be expected to be agonists.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Preparation of MST Clones

A suspension of tumor cells generated from the murine mastocytoma described by Furth, J., et al., Proc Soc Exp Biol (NY) (1957) 95:824-828, which tumor has been passaged over the past thirty years in mice and continues to produce heparin in vivo, was treated to remove macrophages and fibroblasts, according to the procedure of Jacobsson, K.G., J Biol Chem (1985) 260:12154-12159, and then treated with streptomycin, penicillin, gentamycin, and Fungizone to remove contaminating bacteria and fungi. The treated cells were cultured for three days with tetracycline in RPMI 1640 growth medium supplemented with 15%

heat-inactivated fetal bovine serum and then maintained in RPMI 1640 growth medium supplemented with 15% heat-inactivated fetal bovine serum and 100 U/ml of penicillin G. The cells were grown under a 5% CO_2/95% air mixture at 37°C.

The cultured cells were stored frozen under liquid nitrogen in growth medium containing 8% glycerol as cryoprotectant, and were designated MST. Subclones of the MST line were generated by limiting dilution in microtiter dishes yielding eight sublines designated MST1-8. These clones and the parental cell population grow as single cells or aggregates in suspension and divide every 12-16 hr. They grow in a variety of growth media, especially well in various preparations of Eagle's medium and Ham's F10 medium containing as little as 0.5% heat-inactivated fetal bovine serum and 1% bovine serum albumin. The parental MST cells are also capable of forming tumors in athymic (nu/nu) Balb/c mice and Leaden x A strain F_1 hybrid mice.

Example 2

Preparation of Antiproliferative Heparin

Heparin was recovered from the parental MST culture by first separating the cells from the growth medium by centrifugation. The cell pellet was washed once with phosphate-buffered saline and solubilized at room temperature for 15 minutes in 0.1 M sodium hydroxide (25 ml/10^9 cells). The viscous solution was adjusted to pH 5.5 with 1.6 equivalents of acetic acid, 0.58 g of NaCl and 50 mg of Pronase (Calbiochem)/10^9 cell equivalents were added, and the sample was incubated overnight at 40°C to degrade cell proteins and the proteoglycan protein cores. Two more portions of Pronase were added over the next two days. The sample was then centrifuged to remove insoluble debris and the pellet was washed twice with 10 ml of 1 M NaCl.

The combined supernatants were diluted to 100 ml with water to lower the salt concentration and chromatographed over a 20 ml bed of DEAE-Sephacel equilibrated in 0.4 M NaCl/20 mM sodium acetate at pH 6.0. The column was washed with 100 ml of 0.4 M NaCl/20 mM sodium acetate/0.2% Zwittergent 3-12 (pH 6.0) to remove lipids and other nonpolysaccharide contaminants, and then with 300 ml of 0.4 M NaCl/20 mM sodium acetate to remove residual detergent. Heparin was eluted with 100 ml of 2 M NaCl/20 mM sodium acetate, dialyzed, and lyophilized. The final salt concentration was 0.1 M NaCl/50 mM sodium acetate, and the solution was adjusted to pH 7.0 and 10 mM $MgCl_2$.

RNA and DNA were digested overnight at room temperature by addition of 10 mg of RNAse A and 1 mg of DNAse I. Chondroitin sulfate was digested by two additions of 0.5 U of chondroitinase ABC (37^oC, 3 hr). Residual peptides covalently bound to heparin were removed by adjusting the solution to 0.5 N NaOH/1 M $NaBH_4$ and incubation at 4^oC for 24 hours.

The solution was neutralized with acetic acid, diluted with water to 40 ml, and chromatographed on a 5 ml bed of DEAE-Sephacel. Nonheparin contaminants were removed with 100 ml of 0.4 M NaCl/20 mM Sodium acetate (pH 6), and pure heparin was eluted with 25 ml of 2 M NaCl/20 mM sodium acetate. The sample was dialyzed against 0.1 M sodium acetate, lyophilized, dissolved in 5 ml of water, and precipitated with 4 volumes of ethanol. The final preparation was dissolved in phosphate-buffered saline and contained no detectable protein or nucleic acid by UV-light adsorption.

The parent MST culture was evaluated for heparin content by the carbazole reaction described by Bitter, T., and Muir, H.M., Anal Biochem (1962) 4:330-334. This uncloned parent culture contained 0.5-1.0 ug heparin/10^6 cells. The derived cloned cell lines MST1-8, wherein the

heparin content was obtained using radioactive sulfate incorporation as described by Esko, J.D., et al., J Biol Chem (1986) 261:15725-15733, contained 0.1-2.0 ug/10^6 cells. The degree of variation of production among the subclones was about a factor of 20.

The recovered heparin was analyzed on anion exchange HPLC using TSK-DEAE-3SW. The elution profile is shown in Figure 1; the position of the eluted material indicates a high level of sulfation. The isolated heparin was subjected to complete digestion in nitrous acid, and the extent of digestion showed that about 80% of the glucosamine residues are N-sulfated.

The relative amounts of heparin/heparan sulfate and chondroitin sulfate produced by clones MST1-8 were determined by digesting radioactive GAGs by chondroitinase ABC, according to the method of Suzuki, S., Meth in Enzymol (1972) 28:911-917. The MST cell lines contained about 10-25% chondroitin sulfate and about 75-90% heparin.

The MST parent cell culture was also evaluated with respect to ability of the secreted heparin to inhibit the proliferation of bovine aortic smooth muscle cells by a modification of the method of Castellot, J.J., Jr., Seminars in Thrombosis and Hemostatis (1987) 13:489-503, as follows.

Solutions to be tested were made up in "complete medium," which is DMEM medium containing 10% fetal bovine serum and penicillin/streptomycin.

Bovine smooth muscle cells (SMC) were isolated from bovine pulmonary artery by the method of Benitz, W.E., et al., J Cell Physiol (1986) 127:1-7. SMC from passages 3-10 were plated at 350-700 cells per well in 96-well microtiter plates in the medium above and allowed to attach for 2-4 hr. The complete medium was replaced with DMEM supplemented with 0.1% fetal bovine serum, and the cells were incubated for an additional 72 hr to arrest

cell growth. The low-serum medium was then replaced with complete medium containing the test samples.

The cells were allowed to grow for up to 7 days with replicate plates sampled at regular intervals. Cell number was determined by removing the medium and washing the cells with phosphate-buffered saline, adding 75-150 ul lysis buffer, and assaying for lactate dehydrogenase (LDH) activity, as described by Brandley, B., et al., J Biol Chem (1987) 262:6431. The activity of LDH is proportional to cell number.

MST parent cell culture heparin inhibited cell growth at 1/15th the concentration required by commercial heparin.

The heparin secreted by the MST parental line was also tested for ability to bind human antithrombin columns, according to the procedure of Hook, M., et al., FEBS Lett (1976) 66:90-93. Less than 1-2% of the total heparin was bound to antithrombin; this suggests that the material may lack anticoagulant activity. This activity is lower than that found in heparin secreted by the mastocytoma tumors from which they originated, which generally contains 15% of high-affinity antithrombin-binding material (Jacobsson, K.-G., et al., J Biol Chem (1985) 260:12154-12159). Commercial heparin generally contains about 30% high-affinity material.

Example 3

Preparation of Adherent (MTA) Cultures and Cell Lines

The MST parent cell culture was grown in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum in plastic cell culture flasks. During growth, a few cells attached to the surface; the un-attached cells were removed from the flask by changing the medium, and the adherent cells continued to proliferate. The medium was changed every 4-7 days, and the monolayer was vigorously washed to remove loosely adherent cells. A

confluent monolayer of firmly attached cells was generated after 4-6 weeks.

The monolayer was treated with 0.1% trypsin and 1 mM EDTA in order to release the cells as a suspension, and the released suspension, designated MTA, was frozen in liquid nitrogen. Samples of the parent culture were subcloned by limiting dilution to obtain the clonal cell lines MTA1-9.

The MTA cell lines were assayed for heparin production using radioactive sulfate incorporation. As with the MST cultures, the radioactive GAGs were about 10-25% chondroitin sulfate and about 75-90% heparin. The total heparin content of the cloned cell lines was about 0.5 ug/10^6 cells and varied by a factor of 2 over the lines.

The heparin obtained from MTA cell cultures showed chromatographic and antiproliferative properties similar to those obtained in the MST culture. Cells from MTA1 formed tumors in athymic (nu/nu) Balb/c mice.

Example 4

Production of Mutants

A suspension containing 3 x 10^5 MTA1 cells was treated with 450 ug/ml of ethyl methane sulfonate. The effectiveness of mutagenesis was assessed by determining the appearance of colonies resistant to ouabain. Stocks having a mutation incidence of more than 10^{-4} were used for identification of strains with altered heparin formation.

In order to assess if the amount and type of heparin was altered by the mutagens, mutagenized stocks which showed the required mutation rate were replica plated and assayed for heparin synthesis. In this procedure, a population of mutagen-treated MTA-1 cells that had been maintained at 33°C in order to rescue strains that bore mutations in essential genes were

harvested with trypsin and EDTA to produce a single cell suspension. About 300 viable cells were added to multiple 100 mm diameter tissue culture plates containing 15 ml of RPMI 1640 growth medium supplemented with 15% heat-inactivated fetal bovine serum and penicillin G. After two days' incubation at 33°C, which allowed the cells to attach to the plate and recover from the trypsin treatment, a disk of 27-micron pore-diameter cloth was placed in the dish. A disk of 1-micron pore-diameter cloth was placed on top of the first disk, and a layer of glass beads were added to weight the disks against the bottom of the dish. The cells, sandwiched between the overlay and the bottom of the dish, were incubated at 33°C, and the medium was changed every 5-7 days. After 14 days, colonies that had formed on the plastic dish had also grown through the polyester disks, as shown by staining of a plate and the pair of corresponding disks with Coomassie blue.

In the first screening, 60 plates were set up in this manner. After colonies formed, the medium was aspirated and the beads decanted from the dish. The disks were then removed from the dish with sterile tweezers. The top 1-micron pore-diameter disk was discarded and the lower 27-micron pore-diameter disk was transferred to a bacterial petri dish filled with RPMI 1640 growth medium supplemented with 15% heat-inactivated fetal bovine serum and penicillin G. The replica disks were incubated overnight at 40°C in order to express any temperature-sensitive mutations that might affect heparin synthesis. The disks were transferred to a fresh bacterial petri dish containing 5 ml of RPMI growth medium supplemented with 15% heat-inactivated fetal bovine serum, penicillin G, and 10 uCi/ml of radioactive sulfate. The disks were incubated at 40°C for four hours, removed from the labeled medium, and treated with a few milliliters of 10% trichloroacetic acid to precipitate any radioactive

heparin. Unincorporated radioactive sulfate was removed by placing each disk on top of a piece of filter paper in a Buchner funnel and washing the disk with 100 ml of 2% trichloroacetic acid. After the disks were dry, they were exposed to X-ray film for 2 days and then they were stained with Coomassie blue. Superimposition of the autoradiograph over the stained disk showed the location of colonies that incorporated less radioactive sulfate than expected from the intensity of the Coomassie blue stain. The low incorporation of radioactive sulfate indicates a deficiency in sulfated GAG synthesis.

When the original polyester overlays were removed from the master dishes, the dishes were overlaid with a fresh polyester disk moistened with growth medium and refilled with RPMI growth medium supplemented with 15% heat-inactivated fetal bovine serum, 2.5 ug/ml Fungizone, and 100 U/ml penicillin. They were incubated at 33°C for 3 days to allow the colonies to fill in on the plate and then shifted to 28°C, which maintained their viability but prevented them from further growth.

After identifying colonies that appeared defective in sulfated GAG synthesis by autoradiography, the medium in the master dishes was replaced with RPMI 1640 growth medium containing 15% heat-inactivated fetal bovine serum and penicillin G, and they were shifted to 33°C for 3 days. The medium and the overlay were then removed from master dishes that corresponded to the disks which the screening indicated had heparin-deficient clones. The pattern of colonies on the disk and the plate were aligned, and the location of defective colonies was circled on the bottom of the plate. The desired colonies were picked with glass cloning cylinders and localized trypsin/EDTA treatment. The cells were diluted and immediately passed through the screening procedure again in order to confirm the mutant phenotype. A portion of the colony was also cloned by limiting dilution in microtiter

plates. Several single-cell clones obtained from the microtiter plates were subjected to the polyester screening assay to determine which clone was defective. Defective purified clones were expanded in cell culture and stored under liquid nitrogen. The details of the screening method and other relevant procedures is given in Esko, J.D., Meth in Cell Biology (1989) 32:387-422, and references therein.

Example 5

Enrichment for Mutants by Radioactive Sulfate Suicide

Clones deficient in sulfated GAG synthesis are relatively rare, occurring with an incidence of about 10^{-4} to 10^{-5}. In order to increase the likelihood of obtaining desirable mutants, a portion of mutagen-treated cells were revived from liquid nitrogen and grown to confluence at 33°C. The culture medium was replaced with RPMI 1640 growth medium that had been made without any inorganic sulfate and supplemented with 15% heat-inactivated fetal bovine serum that had been dialyzed against phosphate-buffered saline to remove inorganic sulfate. The culture was shifted to 40°C overnight and then the medium was replaced with the sulfate-free growth medium described above and 30 uCi/ml of radioactive sulfate. After 4 hours at 40°C, the cells were washed with RPMI growth medium containing normal amounts of inorganic sulfate and then harvested with trypsin/EDTA. The cell suspension was concentrated by centrifugation and resuspended in RPMI 1640 growth medium containing 15% heat-inactivated fetal bovine serum and 8% glycerol. The cells were stored frozen under liquid nitrogen in sealed glass ampules at 2 x 10^6 cells/ml. After 6 weeks, they were thawed, centrifuged to remove the glycerol, and grown for several days at 33°C in normal growth medium. The surviving cells were then passed through the replica-plating screening procedure described in Example 4. The incidence of

sulfated GAG-deficient mutants was found to be 10^{-1} to 10^{-2}, or about 3-4 orders of magnitude more frequent than in the original mutagen-treated population prior to enrichment.

Claims

1. A continuous cell line prepared from murine mastocytoma cells, which cell line continuously secretes over a period of 6 months or more a glycosaminoglycan (GAG) composition consisting of about 15% chondroitin sulfate and about 85% heparin/heparan sulfate, wherein said GAG composition inhibits the proliferation of smooth muscle cells.

2. The cell line of claim 1 which has been selected for adherence to plastic surfaces from a panel of sublines of said murine mastocytoma cells.

3. The cell line of claim 2 which is MTA1, ATCC accession no. _____.

4. A GAG composition produced by the cell line of claim 1 wherein said GAG composition exhibits antiproliferative activity with respect to cultured smooth muscle cells and which GAG lacks antithrombin-binding activity.

5. A GAG composition produced by the cell line of claim 2 wherein said GAG composition exhibits antiproliferative activity with respect to cultured smooth muscle cells and which GAG lacks antithrombin-binding activity.

6. The polysaccharide of claim 4 which is a heparin.

7. The polysaccharide of claim 5 which is a heparin.

8. A method to produce and identify a cell line capable of continuous GAG production, which method comprises

treating a suspension of at least one adherent cell line derived from a mammalian mastocytoma with a mutagen;

plating the treated cells at clonal dilution on a plastic surface to which the cells adhere;

overlaying the surface with at least one absorbent disk; and

detecting the presence or absence of GAG at the location of the resulting colonies on the absorbent disk.

9. The method of claim 8 wherein the disk comprises polyester cloth.

10. The method of claim 9 wherein the disk consists of two layers of polyester cloth wherein the lower layer has a pore size greater than the upper layer.

11. The method of claim 10 wherein the pore size of the lower layer is about 27 microns and the pore size of the upper layer is about 1 micron.

12. The method of claim 8 wherein GAG is detected by incorporation of radiolabeled sulfate.

13. The method of claim 8 which further includes selecting mammalian mastocytoma cells for adherence.

14. A method to screen for mastocytoma cell colonies mutant for GAG biosynthesis and secretion, which method comprises inducing colony formation from single adherent mastocytoma cells on tissue culture plates overlaid with an absorbent layer of polyester cloth so as

to result in transfer of cells derived from the colonies to said cloth; and

assessing the amount and/or nature of GAG synthesized by the transferred colonies.

15. The method of claim 14 wherein the GAG synthesized is assessed by detecting the amount of radiolabeled sulfate incorporated by said colonies.

16. The method of claim 14 wherein the GAG synthesized is assessed by determining its antiproliferative activity.

17. The method of claim 14 wherein the GAG synthesized is assessed by determining its antithrombin-binding activity.

18. The method of claim 14 wherein the GAG synthesized is assessed by determining its antiinflammatory activity.

19. A pharmaceutical composition useful in the treatment of conditions characterized by unwanted smooth muscle cell proliferation, which composition comprises an effective amount of the heparin composition of claim 6 in admixture with at least one pharmaceutically acceptable excipient.

20. A method to treat a condition characterized by unwanted smooth muscle cell proliferation, which method comprises administering to a subject in need of such treatment an effective amount of the heparin composition of claim 6 or a pharmaceutical composition thereof.

21. The method of claim 20 wherein the condition is a result of surgery or trauma.

22. The method of claim 20 wherein the condition is selected from the group consisting of Goodpasture's syndrome, acute glomerulonephritis, neonatal pulmonary hypertension, asthma, acute pulmonary hypertension, and renal vascular hypertension.

23. The method of claim 20 wherein the oligosaccharide composition is administered intravenously.

24. The method of claim 21 wherein the oligosaccharide composition is administered topically or at the location of trauma.

25. Antibodies specifically immunoreactive with the heparin composition of claim 6 and prepared by a process which comprises immunization of a mammal with the said composition.

26. The antibodies of claim 25 which are monoclonal antibodies.

27. The antibodies of claim 25 which are conjugated to label.

28. A method to assay the level of antiproliferative factor in a biological sample, which method comprises treating said sample with the antibodies of claim 25 and detecting the amount of antibodies bound by the sample.

29. A method to enhance smooth muscle cell proliferation in a subject which comprises administering to a subject in need of such enhancement an effective amount of the antibodies of claim 25.

30. A method to prepare antiproliferative heparin agonist antibodies, which method comprises immunization of a mammal with the antibodies of claim 25 and recovery of the agonist antibodies.

31. Antibodies prepared by the method of claim 29.

32. A method to produce cell cultures which are deficient in production of sulfated GAGs which method comprises:

culturing mutagen-treated cells in selection medium containing radioactive sulfate and

recovering cells able to grow in this medium.

33. The method of claim 32 wherein said mutagen-treated cells were treated with ethyl methane sulfonate.

34. The method of claim 32 which further includes identifying sulfated GAG-deficient colonies by inducing colony formation from single adherent mastocytoma cells on tissue culture plates overlaid with an absorbent layer of polyester cloth so as to result in transfer of cells derived from the colonies to said cloth; and

assessing the amount and/or nature of GAG synthesized by the transferred colonies.

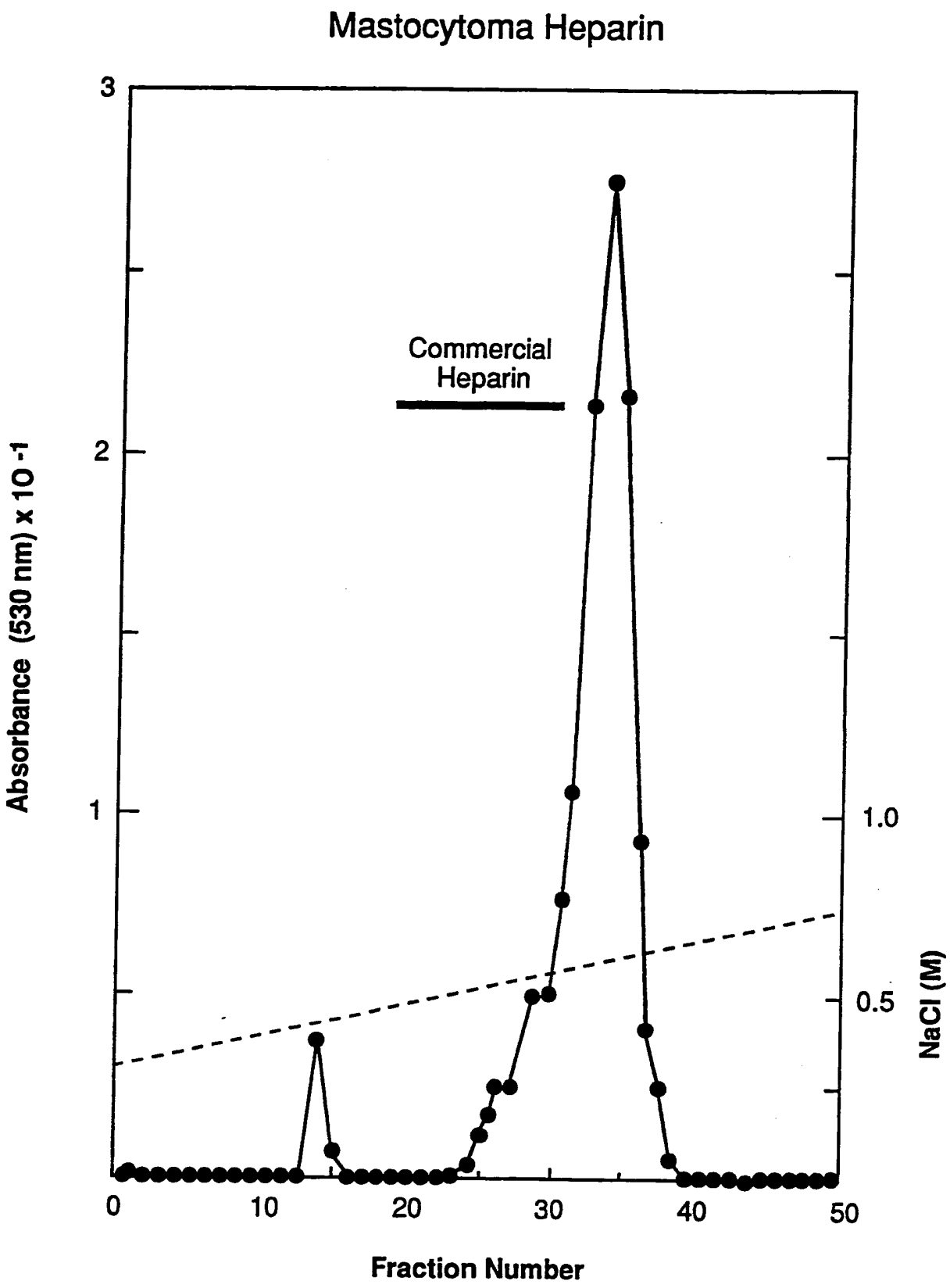
Mastocytoma Heparin
Commercial Heparin
Absorbance (530 nm) x 10 -1
NaCl (M)
Fraction Number
0
10
20
30
40
50
1
2
3
0.5
1.0

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02779

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00; C12Q 1/04; C07H 5/04; A01N 43/04, A61K 39/00
US CL.: 435/240.2, 435/34, 536/55.1, 514/54, 424/85.8

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	536/55.1; 536/55.2; 435/240.2; 435/34; 435/35; 514/54; 514/56 424/85.8

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	THE JOURNAL OF CELL BIOLOGY, VOLUME 107, NUMBER 6 PART 3, PAGE 157a ABSTRACT NUMBER 892, DECEMBER 1988 "REPLICA PLATING OF MURINE MASTOCYTOMA CELLS" MONTGOMERY, et. al. (SEE ENTIRE ABSTRACT)	1 - 3
Y	THE BIOCHEMICAL JOURNAL VOLUME 246, NUMBER 2, PAGES 409-415 01 SEPTEMBER 1987 "DEGRADATION OF HEPARIN PROTEOGLYCAN IN CULTURED MOUSE MASTOCYTOMA CELLS" JACOBSSON, et. al. (SEE PAGE 410, BRIDGE PARAGRAPH OF LEFT AND RIGHT COLUMNS; FIGURE 4, LEGEND)	1 - 3, 8 - 13
Y,P	TRANSPLANTATION PROCEEDINGS VOLUME 21, NUMBER 4, PAGES 3700-3701, AUGUST 1989 "INHIBITION OF SMOOTH MUSCLE CELL PROLIFERATION BY HERPARIN MOLECULES CLOWES, et. al. (SEE PAGE 3700, 2ND PARAGRAPH)	4 - 7, 19-24
Y	FEBS LETTERS VOLUME 66, NUMBER 1, PAGES 90-93, JULY 1976 "ANTICOAGULANT ACTIVITY OF HEPARIN: SEPARATION OF HIGH-ACTIVITY AND LOW-ACTIVITY HEPARIN SPECIES BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED ANTITHROMBIN HOOK et. al. (SEE ENTIRE DOCUMENT)	4-7, 19-24

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
08 AUGUST 1990	30 AUG 1990
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	George Elliot

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y,P	NEUROSURGERY VOLUME 25, NUMBER 6, PAGES 892-898 DECEMBER 1989 "LOCALIZED RELEASE OF PERIVASCULAR HEPARIN INHIBITS INTIMAL PROLIFERATION AFTER ENDOTHELIAL INJURY WITHOUT SYSTEMIC ANTICOAGULATION" OKADA et. al. (SEE TITLE, INTRODUCTION, FIGURES 1 AND 3).	19-21, 24
Y	US,A, 4,824,436, 25 APRIL 1989 "METHOD FOR THE PREVENTION OF PESTENOSIS" WOLINSKY (SEE COLUMN 2, LINES 35-52; ENTIRE DOCUMENT)	4-7,19-21,24
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES (USA), VOLUME 82, PAGES 3197-3201, MAY 1985, "ANIMAL CELL MUTANTS DEFECTIVE IN GLYCOSAMINOGLYCAN BIOSYNTHESIS" ESKO,et.al. (SEE ENTIRE DOCUMENT).	8-13, 32-34

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. A Cell Line (Claims 1-3).
II. Gag or Polysaccharide compositions, Method of Use (Claims 4-7,19-24).
III. Methods of Producing and identifying mutant cell lines (Claims 8-13,32-34).
IV. Method of screening mutant cell lines (Claims 14-18).
V. Antibodies, Methods of using antibodies, Method of making antibodies (Claims 25-31).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y,P	US,A, 4,840,940 20 JUNE 1989 "METHOD FOR REDUCING THE OCCURRENCE OF DISTAL ANASTOMOTIC INTIMAL HYPERPLASIA USING FRACTIONATED HEPARIN" SOITIURAI (SEE COLUMN 3, LINES 30-40; COLUMN 5, LINE 54; COLUMN 6, LINE 28; CLAIMS 1-3, 6-7)	4-7,19-21, 23
Y,P	US,A, 4,883,751 28 NOVEMBER 1989 "SPECIFIC IMMUNOASSAY FOR HEPARIN" GITEL, et. al. (SEE EXAMPLE 4)	25 - 31
L	US,A, 4,849,339 18 JULY 1989 "METHOD FOR DETERMINATION AND DIAGNOSIS OF NON-GOODPASTURE GLOMERULONEPHRITIS" FILLIT, et. al. (SEE DESCRIPTION, COLUMN 1, LINE 18 TO COLUMN 2, LINE 28) REFERENCE SUGGESTS SEVERE CONSEQUENCES OF SYSTEMIC ADMINISTRATION OF ANTIBODIES TO, AT LEAST, HEPARAN SULFATE.	29
Y	CIRCULATION RESEARCH, VOLUME 46, NUMBER 5, PAGES 625-634, MAY 1980, "INHIBITION OF RAT ARTERIAL SMOOTH MUSCLE CELL PROLIFERATION BY HEPARIN" (SEE ENTIRE DOCUMENT).	4 - 7

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